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Antibody Response to Immunization with Purified GD3 Ganglioside and GD3 Derivatives (Lactones, Amide and Gangliosidol) in the Mouse*

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Abstract

GD3 is the ganglioside most abundantly expressed on the cell surface of human melanoma, and treatment with a monoclonal antibody recognizing GD3 has induced major responses in a small proportion of patients. However, we have been unable to induce production of GD3 antibodies in melanoma patients by active immunization with GD3-expressing melanoma cells or purified GD3. In this report we describe attempts to increase the immunogenicity of GD3 in the mouse by chemical modification. GD3 lactone I and II, GD3 amide and GD3 gangliosidol were synthesized, and the humoral immune response to these derivatives was compared with the response to unmodified GD3. The GD3 derivatives were more immunogenic than GD3. At a low dose all congeners induced an IgM response, with antibody titers higher than those elicited by low-dose GD3. The gangliosidol and amide derivatives also induced an IgG response. IgM antibodies induced by immunization with GD3 lactone I cross-reacted with purified GD3 and GD3-expressing melanoma cells. Titers of GD3 cross-reactive antibodies were slightly higher than after immunization with GD3 itself at the same low dose. IgM and IgG antibodies induced by the other congeners did not cross-react with GD3.

Introduction

In studies of the humoral immune response to ganglioside vaccines in patients with malignant melanoma, it has been shown that GM2 is consistently immunogenic, that GD2 elicits an antibody response only occa-

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Abbreviations: The designations GM3, GM2, GM1, GD3, GD2 and GD1b are used in accordance with the abbreviated ganglioside nomenclature proposed by SVENNERHOLM (33). *ELISA* = enzyme-linked immunosorbent assay; *HPTLC* = high-performance thin-layer chromatography; *ITLC* = immune thin-layer chromatography; *IA* = immune adherence; *PA* = protein A.

sionally and that GD3 does not induce production of antibody (1–3). GD3, however, is of interest for vaccine construction because it is the most commonly expressed cell surface ganglioside of human melanomas (4–6) and has been a target for the treatment of melanoma with a monoclonal antibody (7). For this reason, we have attempted to increase the immunogenicity of GD3 by chemical modification, and we have examined the humoral immune response to these GD3 derivatives in the mouse. We report that antibodies raised against GD3 lactone I showed cross-reactivity with GD3 in several assays including reactivity with human melanoma cells expressing GD3, while antibodies induced against GD3 lactone II, GD3 amide and GD3 gangliosidol cross-reacted with GD3 in ELISA but not in the other assays.

Material and Methods

Gangliosides

GM3, GM2, GM1, GD1b and GD3 were provided by Fidia Research Laboratories (Abano Terme, Italy). GD2 was prepared from GD1b by treatment with bovine testis β-galactosidase (8). Gangliosides of the human melanoma cell line SK-MEL-19 were prepared without saponification or peracetylation by published procedures (9, 10).

Ganglioside derivatives

GD3 lactones were prepared by treating calf brain GD3 with glacial acetic acid as described (11). Lactones were separated according to charge by DEAE-Sephadex A-25 chromatography, eluting lactone II in chloroform/methanol/water 30:60:8 v/v/v and lactone I in 0.05 M NH_4Ac in methanol (12). GD3 amide was obtained by aminolysis of GD3 lactone II (13), followed by treatment with 0.05 M NaOH in methanol for 1 h at 37 °C. GD3 gangliosidols were obtained by reduction of GD3 lactone II with sodium borohydride (14). All derivatives were further purified by Sephadex LH-20 chromatography using chloroform/methanol 1:2 v/v as eluent. The structures of GD3 derivatives are shown in Figure 1.

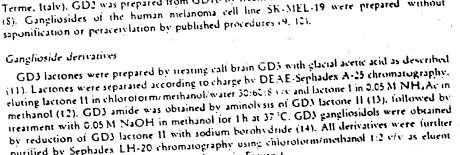

Figure 1. Schematic structures of GD3 derivatives used in these studies. For the proposed structures of GD3 lactones refer to reference (11).

Chemicals

HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); nitrocellulose membranes (0.2 μm) from Schleicher and Schuell, Inc. (Keene, NH, USA), Sep-Pak C_{18} cartridges from Waters Associates (Milford, MA, USA); DEAE-Sephadex A-25, Sephadex G-50 and LH-20, 4-chloro-1-naphthol, and p-nitrophenyl phosphate disodium from Sigma Chemical Co. (St. Louis, MO, USA); cyclophosphamide (Cytoxan) from Mead Johnson (Syracuse, NY, USA).

Monoclonal antibodies and enzymes

Rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase for ITLC were obtained from Dako Corporation (Santa Barbara, CA, USA), rabbit anti-mouse IgM or IgG conjugated with horseradish peroxidase or alkaline phosphatase from Zymed (San Francisco, CA, USA); MAbs R24, C5 and K9 were generated in our laboratory (15). Bovine testis β-galactosidase was obtained from Dr. George W. Jourdian (Michigan State University, Ann Arbor, MI, USA).

High-performance thin-layer chromatography

TLC analysis was performed on HPTLC silica gel plates. Gangliosides and ganglioside derivatives were separated in chloroform/methanol/0.02% aqueous $CaCl_2$ 60:35:8 (v/v) solvent, and visualized by staining with orcinol/H_2SO_4 or resorcinol/HCl. Two-dimensional TLC was performed as described (13).

Immunization

Six-week old female BALB/c × C57BL/6 F1 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were injected intraperitoneally with cyclophosphamide (16, 15 mg/kg 3 days before the first immunization. Gangliosides used for immunization were dried in conical tubes and resuspended in distilled water containing the adjuvant *Salmonella minnesota* rough mutant R 595 prepared as described (17). The mixture was lyophylized and emulsified in PBS prior to administration. Mice were injected subcutaneously with a given ganglioside twice, three weeks apart, at a dose of 10 μg glycolipid and 0.5 mg *S. minnesota* R 595 in 100 μl PBS. Mice were bled from the retro-orbital sinus before and two weeks after the first and second vaccine injection. Serum samples for serological testing were stored at −20 °C.

Dot blot immune stains and enzyme-linked immunosorbent assays

These assays were performed as described previously (10).

Table 1. Immunoreactivity of GD3 derivatives

Derivative	anti-GD3 mabs					
	K9		C5		R24	
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
GD3	++	+++	++	+++	++	+++
GD3 Amide	−	−	−	−	−	−
GD3 Gangliosidol	−	−	−	−	−	−
GD3 Lactone I	−	++	−	++	−[a]	++
GD3 Lactone II	−	+	−	+	−	+

Determined by ITLC; antibody dilution: 20 μg/ml; anti-GD3 antibodies were incubated overnight. Reactivity was grated as follows: +++ strong, ++ moderate, + weak, − not reactive; [a] minute reactivity.

Immune thin-layer chromatography

Immunostaining of gangliosides and ganglioside derivatives with monoclonal antibodies or mouse sera after separation on HPTLC silica gel glass plates was performed as described (18) with minor modifications (19).

Immune adherence and protein A hemadsorption assays

These assays measure antibody mediated rosetting of human RBC (blood group 0) on target cells. Assays were performed as described (22, 23).

Results

Preparation and characteristics of GD3 lactones, amide and ganglioside

Two major products were obtained by treatment of GD3 with glacial acetic acid. Their TLC-patterns (Fig. 2) corresponded with those of GD3-lactone I and GD3-lactone II as described (11). After mild base treatment, both derivatives co-migrated with the parent GD3. After separation on DEAE-Sephadex A-25 according to charge, lactone I was eluted in the monosialo fraction, whereas lactone II was found in the neutral fraction suggesting that one carboxyl group in lactone I and both carboxyl groups in lactone II were involved in the formation of lactone rings. While no other bands were detected by TLC analysis in the lactone II preparation, the lactone I preparation contained 5–10 % GD3. Attempts to remove GD3

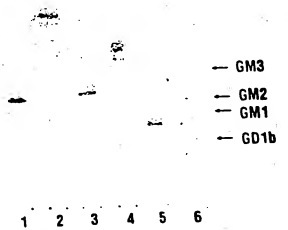

Figure 2. TLC analysis of GD3 and GD3 derivatives used in these studies. GD3 lactone I (Lane 1); GD3 lactone II (Lane 2); GD3 amide (Lane 3); GD3 ganglioside (Lane 4); GD3 (Lane 5); reference gangliosides GM3, GM2, GM1 and GD1b (Lane 6). HPTLC on silica gel plate; running solvent chloroform/methanol/0.22 % aqueous $CaCl_2$ 62:35:8 (v/v); staining reagent: orcinol/H_2SO_4.

completely from this preparation failed, because some GD3 was always formed again during the purification steps as a consequence of the labile nature of the GD3 lactone I structure. After aminolysis of GD3 lactone II two major products migrating as double bands were detected by TLC, one migrating slightly faster than GM3, the other migrating between GM3 and GM2. The faster migrating product was converted to the slower migrating product by mild base treatment, and only the latter was used in our studies. In contrast to lactones, the GD3 amide showed uniform motility in two-dimensional TLC analysis, after being kept in a chamber saturated with ammonia between the two runs. The GD3 amide preparation was free of GD3 and other products as determined by TLC. The product obtained after reduction of GD3 lactone II appeared as a double band in TLC, migrating slightly faster than GM3. This ganglioside preparation contained traces of a product migrating with GD3 lactone II and a double band migrating with GM2 (7%). When stained with resorcinol reagent only the major double band developed the typical orange-yellowish color that has been described for gangliosidols (22).

Reactivity of GD3 lactones, amide and ganglioside with anti-GD3 mAbs

Immune reactivity of these GD3 derivatives with murine mAbs R24, C5 and K9 (recognizing GD3) was determined by ITLC. Results are shown in Table 1. None of the GD3 derivatives reacted with anti-GD3 antibodies when incubated at 4°C overnight. However, both lactones showed some

Table 2. Antibody response of mice after vaccination with GD3 and GD3 derivatives as determined by ELISA*

Vaccine	Dose	No. of mice	Target	Titers	
				IgM	IgG
GD3	0.1 μg	5	GD3	–	–
	1 μg	5	GD3	–	–
	10 μg	20	GD3	40 (3)	–
	50 μg	5	GD3	1280 (1), 320 (1), 80 (1), 40 (1)	–
GD3-L I	10 μg	20	GD3-L I	1280 (2), 640 (1), 320 (1), 160 (2), 80 (2), 40 (9)	–
			GD3	640 (1), 160 (2), 80 (7), 40 (7)	–
GD3-L II	10 μg	15	GD3-L II	640 (1), 160 (1), 80 (3)	–
			GD3	40 (5)	–
GD3-A	10 μg	15	GD3-A	1280 (4), 640 (2), 320 (3), 160 (2), 80 (4)	> 1280 (12), 160 (2), 80 (1) 40 (1)
			GD3	640 (2), 160 (2), 80 (3), 40 (2)	
GD3-OL	10 μg	15	GD3-OL	1280 (6), 640 (3), 320 (2), 160 (1), 80 (1), 40 (1)	> 1280 (7), 320 (1), 160 (2), 80 (2)
			GD3	80 (1), 40 (1)	–

* Reactivity is expressed in reciprocal titers.

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Titers	
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	-
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...), 320 (1),	-
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...(7), 40 (7)	-
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...O)	> 1280 (12);
...(2)	160 (2), 80 (1)
...(2)	40 (1)
...	> 1280 (7), 320 (1),
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Table 3. Antibody response of mice after vaccination with GD3 and GD3 derivatives as determined by dot blot immune stain.

Target	Vaccine[1]									
	GD3		GD3-L I		GD3-L II		GD3-A		GD3-OL	
	Number of reactive mice[2]									
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
GM3	1	-	2	-	-	-	-	-	-	-
GM2	-	-	-	-	-	-	-	-	-	-
GM1	-	-	-	-	-	-	-	-	-	-
GD3	4	-	11	-	3	-	1	-	2	-
GD2	-	-	-	-	-	-	-	-	-	-
GD1b	-	-	-	-	-	-	-	-	1	-
GD3-L I	-	-	11	-	-	-	-	-	1	-
GD3-L II	-	-	-	-	1	-	-	-	-	-
GD3-A	-	-	-	-	-	-	15	15	12	-
GD3-OL	-	-	1	-	-	-	4	-	12	-

[1] Each vaccine contained 10 μg ganglioside.
[2] GD3 and GD3-L I were tested in groups of 22 mice. GD3-L II, GD3-A and GD3-OL in groups of 15 mice.

reactivity when incubated with GD3 antibodies at room temperature overnight. It can not be excluded, however, that this reactivity was due to degradation of GD3 lactone to GD3, which we have shown to occur after overnight incubation at room temperature, but not at 4 °C (23).

Immunogenicity of GD3 and GD3 derivatives in the mouse.

The antibody response of mice after immunization with GD3 or GD3 derivatives was analyzed by ELISA (Table 2), dot blot immune stain (Table 3) and by ITLC (Fig. 3).

GD3: Three of 20 mice immunized with 10 μg GD3 responded with production of low titer IgM antibodies against GD3 as determined by ELISA and dot blot immune stain, but not ITLC. Immunization with 0.1 μg GD3 or 1 μg GD3 did not elicit detectable antibody production, while mice immunized with 30 μg GD3 (a dose previously shown to be immunogenic (24)) responded with production of medium titer IgM antibodies against GD3 as determined by ELISA and dot blot immune stains. No IgG antibodies were detected.

GD3 lactone I: Seventeen of 20 mice immunized with 10 μg GD3 lactone I produced IgM antibodies to GD3 lactone I as detected by ELISA, and eleven of the 20 sera were also reactive by dot blot immune stain. Reactivity of these sera with calf brain GD3 was as follows: ELISA 17/20, dot blot immune stain 11/20. Reactivity with human melanoma-derived GD3 was also detected by ITLC. The sera showed no reactivity with GD3 lactone II or other gangliosides. No IgG antibodies were detected.

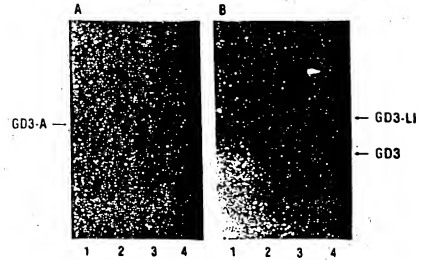

Figure 3. ITLC of mouse antisera induced after immunization with GD3 amide (A) and GD3 lactone I (B). Gangliosides extracted from human melanoma cell line SK-MEL 19 (Lane 1); immunogen: GD3 amide (A) or GD3 lactone I (B) (Lane 2); GD3 from calf brain (Lane 3); reference gangliosides GM3, GM2, GM1 and GD1b (Lane 4). HPTLC on silica gel plates; running solvent chloroform/methanol/0.02% aqueous CaCl$_2$ 60:35:8 v/v; serum dilution 1:75; staining peroxidase and 4-chloro-1-naphthol.

GD3 lactone II: After immunization with 10 μg GD3 lactone II, five of 15 mice developed low titer IgM antibodies against the immunogen and GD3 by ELISA. None of these sera showed reactivity with GD3 lactone II or GD3 by dot blot immune stain or ITLC. These sera showed no reactivity with GD3 lactone I or other gangliosides. Again, no IgG responses were detected.

GD3 amide: Of the GD3 congeners tested, GD3 amide elicited the strongest antibody response. All 15 mice immunized with 10 μg GD3 amide responded with production of high-titer IgM and IgG antibodies reactive with the immunogen as determined by ELISA and dot blot immune stain. Nine of the IgM sera also reacted with GD3 by ELISA, but only one was reactive with GD3 when tested by dot blot immune stain. ITLC revealed only reactivity with the immunogen. IgM antibodies, but not IgG antibodies, showed reactivity with GD3 gangliosidol but not with other gangliosides as determined by dot blot immune stain.

GD3 gangliosidol: After immunization with 10 μg GD3 gangliosidol IgM antibodies to GD3 gangliosidol were detected by ELISA in 14/15 mice and by dot blot immune stain in 12/15 mice, IgG antibodies in 12/15 and 7/15 mice, respectively. Two of the IgM sera also reacted with GD3 in dot blot immune stain tests. Some sera also showed reactivity with GD3 amide by dot blot immune stain and ITLC.

No antibodies were induced in mice immunized with R595 alone as tested by ELISA. The results indicate that these GD3 derivatives are significantly more immunogenic in the mouse than unmodified GD3 or any other ganglioside we have tested (24).

Cell surface reactivity of sera from immunized mice with human melanoma cells

Immune sera were tested for cell surface reactivity with melanoma cells expressing high levels of GD3 (SK-MEL-19), moderate GD3 (SK-MEL-28), or low GD3 (SK-MEL-31). Mice immunized with 0.1 or 1 μg GD3 showed no reactivity, mice immunized with 10 μg GD3 some reactivity, and mice immunized with 30 μg GD3 showed good reactivity (Table 4). Of the GD3 derivatives, only GD3 lactone I induced antibodies reactive with human melanoma cells expressing GD3 on their cell surface. Although some sera from mice immunized with other GD3 derivatives were reactive with GD3 in ELISA, they showed no reactivity with melanoma cells.

Discussion

The ganglioside GD3 is abundantly expressed on the cell surface of melanomas and therefore a potential target for immunological attack. Treatment with a monoclonal antibody-recognizing GD3 has induced regression of melanoma metastases in a small proportion of patients (7). Attempts at inducing an antibody response in melanoma patients by active immunization with GD3 have failed, however (25), and this experience has led us to search for ways in which the immunogenicity of GD3 could be

Table 4. Cell surface reactivity of antisera induced by GD3 and GD3 derivatives in the mouse with three human melanoma cell lines[1]

Vaccine	Dose	Target cell line[2]		
		SK-MEL-19	SK-MEL-28	SK-MEL-31
GD3	0.1 μg	0/5	0/5	0/5
	1 μg	0/5	0/5	0/5
	10 μg	3/20	3/20	0/20
	30 μg	4/5	4/5	1/5
GD3-L I	10 μg	7/20	4/20	1/20
GD3-L II	10 μg	1/15	0/15	1/15
GD3-A	10 μg	0/15	0/15	0/15
GD3-OL	10 μg	0/15	0/15	0/15

[1] Reactivity was determined by IA and PA and is expressed in number of reactive mice immunized with a given vaccine. No reactivity by PA was observed.
[2] Level of GD3 expression on cell surface: SK-MEL-19 high; SK-MEL-28 moderate; SK-MEL-31 low.

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GD3 ganglioside
IA in 14/15 mice
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increased. Several reports indicate that chemical modification may augment the immunogenicity of ganglioside molecules. Ganglioside derivatives that have been previously reported to induce antibody production more readily than the parent molecules include GM1 methylester, GM1 gangliosidol and GM1-N-methylamide (26, 27), GM3 lactone (14, 28) and O-acetylation products of GD3 (10).

We report here that modifications of GD3 resulting in enhanced immunogenicity include the lactone, amide and gangliosidol congeneres. The changes in the molecular structure of GD3 involve loss of charge and altered configuration, hydrophobicity, rigidity and stability. Which of these changes are involved in enhancing immunogenicity is not known. One feature of structurally modified gangliosides is their reduced susceptibility to enzymatic action (26, 29, 30). Higher immunogenicity might result from greater resistance to metabolic degradation, making a lower dose of a GD3 derivative equivalent to an higher dose of GD3. Alternatively, increased immunogenicity might be the consequence of presenting molecular conformations not known to be expressed in mammalian tissue. We have found that the differential expression of individual gangliosides in normal tissues of mice and humans is inversely proportional to their ability to elicit antibody production in these two species (24), suggesting that normal tissue expression rather than chemical structure determines ganglioside immunogenicity.

We were initially impressed by the high-titer antibodies against GD3 detected by ELISA after immunization with some GD3 derivatives (Table 2). However, further testing by dot blot immune stain on nitrocellulose or immune thin-layer chromatography on silica gel plates showed no reactivity with GD3. In our hands, results of dot blot immune stains on nitrocellulose and immune thin-layer chromatography on silica gel plates correlated much better with cell surface reactivity than results of ELISA. Others have also reported that ganglioside antisera show reactivity in ELISA on plastic surfaces but not in other types of tests (31). It appears that epitopes expressed by purified gangliosides in ELISA wells are not necessarily the same epitopes expressed on other artificial matrices or on the cell surface.

In our studies, antisera raised with GD3 amide or GD3 gangliosidol were highly specific for the respective immunogen and showed almost no reactivity with GD3, while antibodies induced by GD3 lactone I (but not lactone II) were equally reactive with GD3 lactone I and with unmodified GD3. Furthermore, these antibodies were reactive with human melanoma cells expressing GD3 on their cell surface, but not reactive with melanoma cells not expressing GD3. Only the GD3 lactone I configuration appeared to be close enough to that of GD3 to induce a crossreactive immune response. Similar observations have been made with GM3. Yu et al. have suggested that the increased hydrophobicity and the more rigid structure of GM3 lactone as compared with native GM3 might favor immunological

recognition (32), and Norris et al. (14, 28) have shown that an immune response against GM3 could be induced with GM3 lactone but not with unmodified GM3.

Our findings suggest that chemical modifications of melanoma gangliosides may increase their immunogenicity and that some chemically modified melanoma gangliosides can be used to construct immunogenic vaccines for immunization of patients with melanoma. We need to keep in mind, however, that the human immune system may not recognize the same epitopes that are recognized in the mouse. Studies investigating the immune response of patients with melanoma to immunization with GD3 derivatives are now underway.

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